REMARKS

Upon entry of this amendment, claim 1 will be amended, with claims 1, 2, 9 and 12 being independent claims. Claims 1-14 are pending, with claims 1-8 being under consideration, and claims 9-14 withdrawn from consideration.

Applicants thank the Examiner for conducting a telephone interview with Applicants' representatives on October 13, 2003. During the interview, the restriction requirement and the art-based rejections of record were discussed. Applicants representatives explained the differences between the cited references and the claimed invention. The Examiner appeared to agree that the claimed invention differed from the prior art, but indicated that she will further review the claims upon receipt of Applicants' response. The arguments presented during the interview to distinguish the claimed invention from the prior art are discussed herein in more detail. Possible amendments to further clarify Applicants' claimed invention to include separation in the claims were also discussed.

Reconsideration and allowance of the application are respectfully requested.

RESPONSE TO FORMAL MATTERS

Information Disclosure Statement

Applicants thank the Examiner for returning an initialed copy of the Information Disclosure Statement filed May 28, 2003.

Drawings

Applicants thank the Examiner for indicating that the drawings filed with the instant application have been deemed to be acceptable.

RESPONSE TO MAINTAINING OF RESTRICTION REQUIREMENT

The Office Action has maintained the Restriction Requirement acknowledging Applicants' election with traverse of Group I and a membrane-bound enzyme, and the requirement has been made final withdrawing claims 9-14 from consideration as being directed to non-elected inventions.

The Office Action contends that membrane-bound enzyme is not directed to a species, and that each of the proteins listed in the claims are also patentably distinct inventions. However, Applicants respectfully submit that each of the proteins is a membrane-associated protein and should be considered to be a species which are rejoinable upon allowance of the elected invention.

Applicants note that during the interview, after Applicants representatives explained the claimed invention, the Examiner agreed that the non-elected proteins of claim 1 would be rejoinable upon allowance of a generic claim.

In view of the above, reconsideration and withdrawal of the restriction requirement or rejoinder of the non-elected subject matter is respectfully requested.

RESPONSE TO REJECTIONS BASED UPON PRIOR ART

The Office Action maintained the rejection of claims 1-8 under 35 U.S.C. 102(b) as being clearly anticipated by Bandman et al. (hereinafter "Bandman"), U.S. Patent No. 5,858,750. The Office Action also maintained the rejection of claims 1-8 under 35 U.S.C. 102(b) as being clearly anticipated by Ikeda et al. (hereinafter "Ikeda"), PNAS, 1995; 92: 126-130.

The Office Action rejected Applicants' prior arguments, and reasserted that Bandman teaches a method of producing a membrane enzyme, human retinol dehydrogenase type II homolog (HRoDH), by culturing a host cell (insect cell baculovirus system) and recovering the enzyme from the host cell culture. The Office Action also rejected Applicants' arguments with respect to Ikeda and maintained the assertion that Ikeda teaches a method of expressing and purifying a membrane-bound enzyme, human γ-glutamyl transpeptidase mutant, by recovering recombinant baculovirus from insect cells. The Office Action held that these teachings clearly anticipated Applicants' claimed invention.

During the telephonic interview of October 13, 2003, Applicants' representatives discussed Applicants' claims, the teachings of both Bandman and Ikeda and then noted differences between the prior art and the claimed invention.

In particular, Applicants' representatives noted that the claimed invention is based upon the discovery that when cells are transformed by a membrane-associated protein, such as those recited in claim 1, using a budded baculovirus system, the budded baculoviruses express the recombinant membrane-associated protein. Applicants discovered that the budded baculoviruses could be separated, and these budded baculoviruses contained the protein of interest. There is

no teaching or suggestion in the prior art of Applicant's invention. Instead, the prior art merely discloses, at most, classic protein purification steps including adding detergents to the cell culture to disrupt all cell wall structures and then purifying the protein of interest.

Applicants respectfully submit that Bandman teaches a human retinol dehydrogenase type II homolog (hRoDH) and states generally that it can be expressed in cells using any of a number of listed expression vectors, including a baculovirus system. Bandman does not discuss Applicants' method to obtain the hRoDH from budded baculoviruses. Indeed, Bandman does not even once state that a baculovirus system may result in budded baculoviruses. Applicants representatives pointed out that Bandman could not teach that the budded baculovirus would express the recombinant protein, nor does Bandman teach a method of collecting those budded baculovirus, especially where it does not even discuss budding at all. Thus, as discussed during the above-noted interview, Bandman is a general teaching which fails to teach, or suggest the limitations of Applicants' claimed invention.

With respect to the rejection of claims 1-8 under 35 U.S.C. 102(b) as being clearly anticipated by Ikeda, Applicants respectfully submit that Ikeda discloses a classic molecular biology deletion experiment whereby a gene of interest is used to transform a cell, the transformed cell is cultured, then the cells are disrupted to obtain and purify the protein. Ikeda uses a baculovirus expression vector system to transform the cells with the gene of interest, γ-glutamyl transpeptidase. However, Ikeda does not discuss the budding of the baculovirus. Instead, Ikeda teaches that the transformed cells were "harvested" after transformation, then treated with several compounds which serve to disrupt cell wall structures to obtain and purify

the recombinant γ-glutamyl transpeptidase. There is no discussion of the activity of the recombinant γ-glutamyl transpeptidase being found in a budded baculovirus, and there is no teaching of separating the budded baculoviruses to obtain the recombinant protein of interest.

In contrast to Ikeda, Applicants discovered that when membrane-associated proteins are used to transform cells using a budding baculovirus system, the budded baculoviruses themselves will express the protein of interest. The Examiner agreed that this result was not appreciated by Ikeda, and that a method of preparing a protein by separating budded baculoviruses was not taught by Ikeda. The Examiner agreed that Ikeda was a more classic teaching which fails to appreciate or teach the limitations of Applicants' claimed invention.

Applicants have included a copy of Urano, Y. et al., A Novel Method for Viral Display of ER Membrane Proteins on Budded Baculovirus, BBRC 308, 191-196 (2003) and Masuda, K. et al., A Combinatorial G Protein-coupled Receptor Reconstitution System on Budded Baculovirus, JBC 278:27, 24552-24562 (2003), two recent articles describing in further detail Applicants' invention to help the Examiner better understand the differences between Ikeda and Bandman and the claimed invention.[1] In particular, the Examiner's attention is directed to page 191-2 of Urano, and pages 24552-4 of Masuda, which discuss the state of the art and the findings of the inventors that membrane associated proteins are expressed in the budded baculovirus itself.

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[1] In accordance with MPEP 609(c)(3), these documents are being submitted as evidence directed to an issue of patentability raised in an Office Action for consideration in connection with arguments being made in reply to the Office Action. Therefore, the requirements of 37 C.F.R. 1.97 and 37 C.F.R. 1.98 need not be satisfied in order to have the documents considered.

In view of the above, the rejections of record are without appropriate basis, and should be withdrawn.

CONCLUSION

In view of the foregoing, the Examiner is respectfully requested to reconsider and withdraw the rejection of record, and allow all the pending claims.

Allowance of the application is requested, with an early mailing of the Notices of Allowance and Allowability.

If the Examiner has any questions or wish to further discuss this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,
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A novel method for viral display of ER membrane proteins on budded baculovirus

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Abstract

The baculovirus expression system has been used to express large quantities of various proteins, including membrane receptors. Here, we reveal a novel property of this expression system to be that certain membrane proteins can be displayed on the budded virus itself. We introduced the genes encoding sterol regulatory element-binding protein-2 (SREBP-2) or SREBP cleavage-activating protein (SCAP), important integral membrane proteins of the endoplasmic reticulum (ER) and/or the Golgi apparatus related to cellular cholesterol regulation, into a baculovirus vector. When insect cells were infected with SREBP-2 or SCAP recombinant viruses, it was found that these ER membrane proteins appeared on the budded baculovirus in addition to the host cell membrane fraction. Compared to proteins expressed on the cell membrane, membrane proteins displayed on virus exhibited both less aggregation and less degradation upon immunoblotting. Using this viral displayed SCAP as the screening antigen, we then generated a new monoclonal antibody specific against SCAP, which was useful for immunological localization studies. This system, which takes advantage of the viral display of membrane proteins, should prove to be a powerful additional tool for postgenomic protein analysis.

Keywords: SREBP; SCAP; Endoplasmic reticulum; Sf9; Baculovirus; Budded virus; Monoclonal antibody

Membrane proteins are one of the major targets for drug discovery in the postgenomic era [1]. The integral membrane proteins play a central role in biological systems as diverse as energy production, intracellular signal transduction, and intercellular communication. For example, the G-protein-coupled receptors (GPCRs), which exist on the plasma membrane, constitute a family of membrane receptors which contribute to many cellular effects through the signal transduction triggered by their binding of extracellular ligands [2].

In another vein, SREBP-2 and SCAP are ER membrane proteins which are thought to play an important role in cellular cholesterol homeostasis [3,4]. SREBP-2 exists on the ER membrane in the form of precursor proteins and complexes with SCAP. SCAP, a polytopic membrane protein, plays a dual role as an escort protein and as a sterol sensor. In sterol-depleted cells, SCAP escorts SREBP-2 to the Golgi apparatus where two sequential proteolytic events release the NH_2-terminal mature form of SREBP-2 into the cytoplasm [4–6]. Then, the mature SREBP-2 enters the nucleus and enhances the transcription of genes encoding enzymes which regulate cholesterol synthesis.

To investigate membrane proteins by biochemical analysis, it is important to exogenously express sufficient amounts of the target functional membrane proteins. Various expression systems have been tried in this quest

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for sufficient expression, but to date a suitable system for the expression of membrane proteins has not been established because of their hydrophobic nature [7–9]. Among the systems tried, baculovirus and insect cells have been commonly used for high quantity expression for a number of different proteins, not only soluble proteins but also membrane proteins as well [10–12]. The baculovirus expression system has several advantages, including posttranslational modifications such as fatty acid acylation and phosphorylation, which do not normally occur in *Escherichia coli*. It may be that the yeast cell wall interferes with the recovery of the expressed protein on a large scale and the insect cell is easier to handle. However, it is also reported that a drawback of high-level expression is that as the immature protein is expressed, it causes massive protein aggregation or even degradation in some cases [10–12].

Loisel et al. [13] have reported that functional β2-adrenergic receptors were recoverable from extracellular virus particles when *Spodoptera Frugiperda* (Sf9) cells were infected with β2-adrenergic receptor recombinant baculovirus. Masuda et al. [14] showed that a trimeric G-protein and BLT1, a leukotriene B4 receptor, could be functionally reconstituted on the budded virus by co-infection of each with recombinant virus in Sf9 cells. Thus, the membrane receptors, which reside on the plasma membrane, were functionally displayed on the budded form of the baculovirus.

Here, we show that the ER membrane proteins are also expressed on budded virus in addition to the Sf9 cells. In contrast to proteins expressing in Sf9 cells, viral displayed proteins were less aggregated and less degraded. And as mentioned already, this suggests an application to screening for specific monoclonal antibody generation using this viral display system.

Materials and methods

Constructs. To construct the baculovirus vectors expressing human SREBP-2 or SCAP (full-length or amino acids 799–958), the following steps were used. pBlueBac3-BP2 was prepared; first, the 5′ half fragment of SREBP-2 was prepared by digesting a plasmid, in which a linker containing a *Spe*I site was added just before the initiation codon of SREBP-2 (1–481) in pOP13BP2 [15], with *Spe*I and *Bcl*I. Secondarily, the 3′ half fragment was prepared by digesting a plasmid containing 151–1141 of SREBP-2 with *Bcl*I and *Cpo*I. The fragments were cloned into pBlueBac3 (Invitrogen) in which *Cpo*I site was introduced between *Bam*HI and *Hin*dIII sites. pBlueBacHis2-SCAP or pBlueBacHis2-SCAP-C was generated by cloning an *Eco*RI/*Hin*dIII or *Xho*I fragment based on KIAA0199 (a gift from the Kazusa DNA research institute) into pBlueBacHis2 (Invitrogen).

Production and purification of recombinant virus and Sf9 cell culture. Sf9 cells were cultured in Grace's Insect Media supplemented (Gibco-BRL) with 10% fetal bovine serum (FBS, Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco-BRL) at 27 °C. Large scale culturing was performed with the addition of 0.001% pluronic F-68 (Gibco-BRL). The generation of recombinant viruses was performed according to the manufacturer's method (Bac-N-Blue Transfecton Kit, Invitrogen). Briefly, cells were transfected with Bac-N-Blue DNA and 4 μg of recombinant transfer vector. Recombinant virus was isolated by successive rounds of plaque assay. After the generation of high titer stock, the viral titer was also determined by plaque assay.

Immunoblotting for expression level of SREBP-2. Sf9 cells (0.83×10^6 cells/6 well dish) were infected with the recombinant virus at a "multiplicity of infection" (MOI) level of 5. Sf9 cells were scraped and centrifuged at 800*g* for 10 min after culturing for 24, 48, or 72 h. Then, a pellet was obtained as the cell fraction and a culture supernatant was obtained. The cell fraction was suspended in 100 μl/well of isotonic buffer (PBS, phosphate buffered saline, containing 0.1% Triton X-100, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 100 μg/ml PMSF, phenylmethylsulfonyl fluoride). After vortexing at 4 °C for 30 min and centrifugation at 1000*g* for 10 min, 20 μl of 5× SDS sample buffer was added to 80 μl of the supernatant, followed by heat treatment at 95 °C for 10 min. The culture supernatant (80 μl) was added to 5× SDS sample buffer (20 μl), and the mixture was subjected to heat treatment. Those samples were applied to 8% SDS-PAGE and transferred to a nitrocellulose membrane (Highbond ECL, Amersham). After blocking with Block Ace for 30 min, the membrane was immunoblotted with monoclonal antibody IgG-1C6 (anti-human SREBP-2 antibody, ATCC No. CRL-2224). Immunoreactive proteins were detected by SuperSignal West Dura (Pierce).

Preparation of budded virus and sucrose density gradient centrifugation. Sf9 cells (5×10^8 cells/500 ml) were infected with SREBP-2 recombinant virus at a MOI of 5 and cultured for 48 h. The cells were removed by centrifugation at 800*g* for 10 min, and then the supernatant was ultra-centrifuged at 40,000*g* for 20 min. The precipitate was suspended in 4 ml of a TE buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA). The suspension was overlaid onto a 25–56% linear sucrose density gradient and centrifuged at 100,000*g* for 90 min in a SW28 rotor. Fractions (1.5 ml) were collected from the top of the gradient, and the distribution of SREBP-2 was examined by immunoblotting with IgG-1C6 and SDS–PAGE with Coomassie brilliant blue staining.

Production of monoclonal anti-human SCAP antibody. Sf9 cells (5×10^8 cells/500 ml) were infected with SCAP-C recombinant virus at a MOI of 5 and cultured for 48 h. The cells were harvested and lysed by sonication, and then the lysate was centrifuged at 10,000*g* for 30 min. The supernatant was applied to a Ni–NTA column (QIAGEN) and the recombinant proteins were purified as an antigen by standard method. Six-week-old female BALB/c mice were immunized three times. Primary ELISA screenings were performed by standard method using purified SCAP-C as the capture antigen. To check the reactivity to full-length SCAP, SCAP (full-length) expressing viruses underwent SDS–PAGE and were transferred to nitrocellulose membranes. Each culture supernatant of positive clones on ELISA was probed to a strip of the transferred membrane.

Preparation of microsomal membranes from several cell lines or rat tissues. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma)/5% FBS/penicillin (100 U/ml) and streptomycin (100 μg/ml). CaCo-2 cells were grown in DMEM/5% FBS/1% non-essential amino acids (Gibco-BRL)/penicillin (100 U/ml) and streptomycin (100 μg/ml). CHO cells were maintained in Ham-F12 (Sigma)/5% FBS/penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured at 37 °C in a 5% CO_2. The cells were suspended in buffer A (10 mM Hepes–KOH at pH 7.4, 0.25 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 5 μg/ml aprotinin, pepstatin A, leupeptin, 2 mM PMSF, and 25 μg/ml *N*-acetyl-Leu-Leu-norleucinal), passed through a 27 G needle 10 times, and centrifuged at 1000*g* for 10 min at 4 °C. The 1000*g* supernatant was ultra-centrifuged at 100,000*g* for 1 h to prepare the microsome fraction and the following pellet was resuspended in SDS sample buffer.

Twelve-week-old Wistar rats were used for all experiments. Liver and testis tissues were homogenized in buffer A with a Teflon homogenizer, and the microsome fraction was prepared by the above-mentioned sequential centrifugation.

Immunohistochemistry. Immunohistochemistry was performed as described [16]. Briefly, rat tissues were fixed in 10% formalin, dehydrated with alcohol, and embedded in paraffin. Tissues were sectioned in 4 μm thick samples. After blocking of endogenous biotin, tissues were incubated for 2 h at room temperature with a monoclonal antibody against SCAP (K7623) which was diluted in 1% BSA/PBS to a final concentration of 10 μg/ml. After several washes with PBS, the sections were incubated with anti-mouse IgG for 1 h and were stained with an avidin–biotin kit according to the manufacturer's protocol (Vectastain Elite ABC kit, Vector, CA). The sections were then counterstained with hematoxylin.

Results

Expression of SREBP-2 on budded virus

By using IgG-1C6, a SREBP-2 specific antibody, the expression level of human SREBP-2 in Sf9 cells was determined via immunoblotting (Fig. 1). After infection with SREBP-2 recombinant virus, SREBP-2 was detectably expressed at 24 h in Sf9 cells. In addition, we detected an intense anti-SREBP-2 reactive band in culture supernatant after 48 h.

To clarify whether this band was derived from cell debris or not, we fractionated the culture supernatant from the 48-h infection timepoint by serial centrifugations. The culture supernatant was centrifuged at 800*g* for 10 min to separate cell debris as a pellet. Then, the supernatant was ultra-centrifuged at 40,000*g* for 20 min. The anti-SREBP-2 reactive band was recovered in the supernatant of the 800*g* centrifuged sample and also recovered in the pellet fraction of a 40,000*g* centrifuged sample (Fig. 2). These results suggest that the SREBP-2 present in the culture supernatant is not debris such as dead cells, but rather is derived from membrane or extracellular virus. To exclude the possibility that it was a contaminant of the cell membrane, the pellet fraction was fractionated by sucrose density gradient centrifugation. As a result, SREBP-2 was observed in the same fraction as that of the virus envelope protein gp64, a finding which was confirmed by SDS–PAGE with Coomassie brilliant blue staining (Fig. 3). This result indicates that the SREBP-2 observed in the ultra-centrifuged pellet fraction is not debris of cell membrane debris, but rather is SREBP-2 expressed on budded virus.

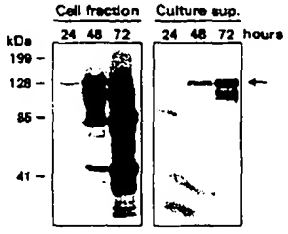

Fig. 1. Expression of SREBP-2 in Sf9 cells and culture supernatants. Sf9 cells were infected at MOI 5, and cultured for 24, 48, or 72 h. The cell fraction and culture supernatant were prepared as described in *Materials and methods*. Aliquots were separated by SDS–PAGE (8%) and were subjected to immunoblotting with anti-SREBP-2 (IgG-1C6). The arrow indicates SREBP-2.

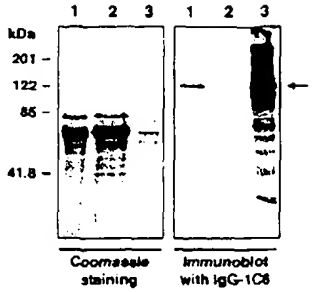

Fig. 2. Centrifugal separation of SREBP-2 expressed in culture supernatant. The culture supernatant from 48 h of infection was fractionated by serial centrifugation. Aliquots were separated by SDS–PAGE (8%) and were subjected to Coomassie brilliant blue staining or immunoblotting with IgG-1C6. Lane 1, 800*g* 10 min supernatant; lane 2, 40,000*g* 20 min supernatant, and lane 3, 40,000*g* 20 min pellet. Arrow indicates SREBP-2.

Expression of SCAP on budded virus

We checked for the expression of human SCAP, which has eight membrane-spanning domains, on budded virus. Similar to SREBP-2, SCAP was also displayed on budded virus after 48-h infection (Fig. 4). In the immunoblotting with anti-His-tag antibody, we noticed that SCAP expressed in the Sf9 membrane largely consisted of high molecular weight immunoreactive bands, presumably aggregates of SCAP. SCAP in the

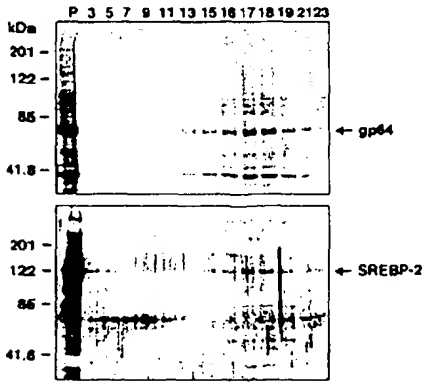

Fig. 3. Sucrose density gradient centrifugation of the pellet fraction of the 40,000*g* centrifuged sample. Aliquots of the gradient fraction were analyzed by Coomassie brilliant blue staining (above panel) and immunoblotting with IgG-1C6 (bottom panel). *P* denotes the pellet fraction of the 40,000*g* centrifuged sample. Fraction numbers are denoted at the top of the panel. Arrows indicate gp64 and SREBP-2, respectively.

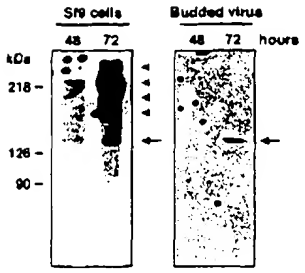

Fig. 4. Expression of SCAP on budded virus. Sf9 cells were infected at MOI 5 and cultured for 48 or 72 h. The cell fraction and viral fraction were prepared by the same method as in Fig. 2, and immunoblotting was performed with an anti-His-tag antibody (Qiagen). The arrow indicates SCAP and the arrowheads indicate high molecular weight immunoreactive proteins, respectively.

budded virus fraction appeared as a single major band with the proper or expected molecular weight.

Application of baculovirus-expressed SCAP as a screening tool for antibody generation

We next evaluated whether proteins expressed on budded virus could be used as a screening tool for antibody generation. SCAP is a polytopic membrane protein which has 8 transmembrane domains in its amino-terminal region. The carboxyl terminal region of SCAP contains WD repeats for interaction with SREBP and projects into the cytoplasm [17]. Therefore, we constructed a baculovirus expression vector which included SCAP carboxyl terminal sequences (799–958 amino acids) with his-tag at the amino terminus. After infection with recombinant virus at a MOI of 5, Sf9 cells were incubated for 48 h and cell lysates were recovered. Recombinant proteins were purified with a Ni–NTA column and were then immunized in mice.

After selection with ELISA using purified SCAP-C as the capture antigen, we performed an immunoblotting screening with SCAP (full-length) expressed virus as described in Materials and methods. As shown in Fig. 5A, it was found that several clones had raised antibodies which recognized full-length SCAP expressed on budded virus.

To determine whether the generated antibody recognized intrinsic SCAP, cell extracts from HepG2, CaCo-2, and CHO cells were immunoblotted with the purified antibody (K7601). Total microsomal fractions prepared from the liver and testis of rat were also tested (Fig. 5B). The monoclonal antibody against human SCAP (K7601) recognized one main band which had the expected molecular size of SCAP in HepG2 and CaCo-2 cells. K7601 also cross-reacted, with a major single band with the expected molecular weight for SCAP, in the liver and testis of rat as well as in CHO cells (Fig. 5B).

Immunohistochemical localization study in rat tissues

Using the anti-SCAP antibody, an immunohistochemical study was performed with rat tissues. Among the tissues tested, the testis and adrenal gland were positively stained. In the testis, the cytoplasm of the interstitial cells, known as Leydig cells, were powerfully stained. In the adrenal gland, the cells in the zona fasciculata and zona reticularis were positively stained. The positive staining of the capsule is considered to be a nonspecific staining frequently seen at the edge of the specimen. These results suggest that SCAP localizes abundantly in the cytoplasm of steroid hormone generating tissues (Fig. 6).

Discussion

A baculovirus system in Sf9 cells enables high-level expression by means of a potent promoter of a baculoviral polyhedrin gene. This system has many advanta-

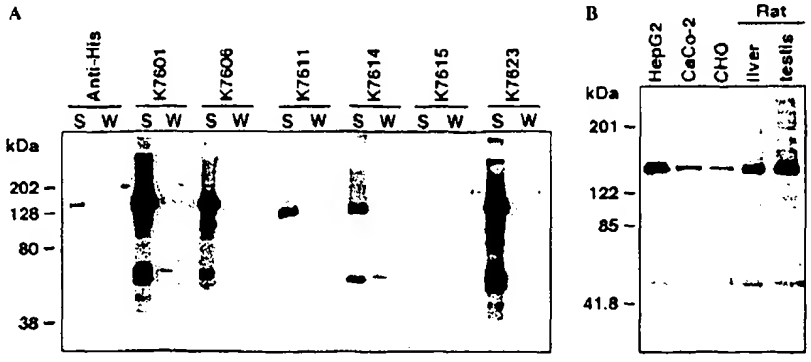

Fig. 5. Screening and specificity of monoclonal antibody against human SCAP. (A) The SCAP (full-length) recombinant virus (S) and wild-type virus (W) were immunoblotted with the culture supernatants of positive clones by ELISA, or with an anti-His-tag antibody as a positive control. (B) Specificity of K7601 was analyzed using HepG2, CaCo-2, and CHO cells, and rat tissues (liver and testis). The microsome fractions (30 μg/lane) were subjected to immunoblotting.

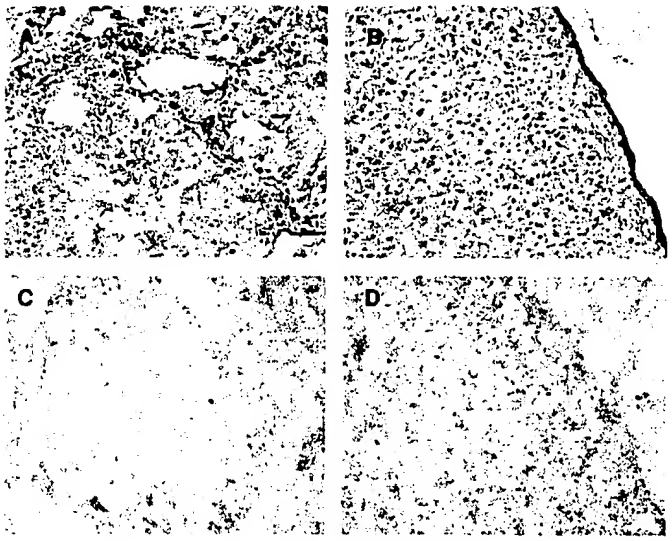

Fig. 6. Immunohistochemical localization of SCAP in rat tissues. Twelve-week-old Wistar rats were treated with an anti-SCAP antibody (K7623). SCAP was localized especially to Leydig cells in testis (A), and zona fasciculata, as well as the zona reticularis cells of adrenal gland (B). (C) and (D) Negative controls, staining without the primary antibody for (A) and (B), respectively.

ges over that of *E. coli*, such that the expressed proteins do not easily aggregate, and do undergo the posttranslational modification which is necessary for protein function.

About 48 h after infection, baculovirus assembles into a budded form and buds on the outside of the insect cells. Utilizing this characteristic, it has been shown that foreign protein is displayed on the viral surface by fusing with gp64, which is a viral structural transmembrane protein [18–21]. Bouvier et al. [22] have reported that a certain seven-transmembrane domain type receptor is expressed not only on the cell membrane but also on budded virus. They reported that the receptors recovered from the viral envelope are functional in comparison to the non-functioning receptors recovered from the cell surface, ostensibly because the majority of receptors on the virus have undergone sufficient posttranslational modification [13,22]. We found in this study that integral membrane proteins distributed on ER or Golgi apparatus were also expressed on budded virus. The membrane proteins displayed on budded virus exhibited either less degradation (Fig. 2) or less aggregation (Fig. 4). These observations imply that these ER membrane proteins are expressed properly on the viral membrane, as in the case of the other membrane receptors.

The precursors of SREBP-2 and SCAP are considered to form heterodimer complexes and reside in the ER in mammalian cells [3]. When the cellular sterol level decreases, SCAP escorts SREBP-2 to the Golgi apparatus where SREBP-2 is successively cleaved by two processing enzymes [5]. After liberation of mature SREBP-2, SCAP returns to the ER [6]. Although it can not be excluded that SREBP-2 and SCAP are inserted in the plasma membrane in Sf9 cells, our observations suggest the possibility that the baculovirus utilizes the ER or Golgi membrane as its envelope, at least for most part, rather than the plasma membrane.

An application toward the generation of monoclonal antibodies was also evident. In general, monoclonal antibodies against membrane proteins are difficult to produce because of certain conformational characteristics. In this study, we used the budded virus as a screening antigen in immunoblotting, so that a specific monoclonal antibody was obtained which could be used not only in immunoblotting (Fig. 5) but also in immunohistochemistry (Fig. 6). It is believed that the conformation of the expressed protein on the budded virus is similar to that of endogenous protein. Therefore an antibody screened by this method would be useful for immunohistochemistry analysis.

By immunoblotting, this anti-SCAP antibody was shown to recognize one major band, which had a molecular weight appropriate for SCAP (Fig. 5). Using this anti-SCAP monoclonal antibody, the immunohistochemical localization of SCAP was studied in various rat tissues. Among the samples tested, interstitial cells of Leydig in the testis and zona fasciculata, as well as the zona reticularis cells of the adrenal gland, which are known to be a major steroidogenic cells, were strongly stained (Fig. 6). On the other hand, the liver was only weakly positive (data not shown). Those observations are compatible with the data on tissue distribution of SCAP mRNA obtained by DNA microarray analysis (available at http://www.lsbm.org/db/index.html).

This study demonstrates that membrane proteins embedded in the membrane of ER or Golgi apparatus are displayed on budded virus when those proteins are expressed using a baculovirus expression system. It was further demonstrated that a system utilizing the baculovirus display system of membrane proteins is a highly useful tool for monoclonal antibody screening.

Acknowledgments

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A Combinatorial G Protein-coupled Receptor Reconstitution System on Budded Baculovirus

EVIDENCE FOR $G\alpha_i$ AND $G\alpha_o$ COUPLING TO A HUMAN LEUKOTRIENE B_4 RECEPTOR*

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To investigate the coupling selectivity of G proteins and G protein-coupled receptors (GPCRs), we developed a reconstitution system made up of GPCR and heterotrimeric G proteins on extracellular baculovirus particles (budded virus (BV)). BV released from Sf9 cells infected with a recombinant baculovirus coding for human leukotriene B_4 receptor (BLT1) cDNA exhibited a high level of BLT1 expression (27.3 pmol/mg of protein) and specific [^{3}H]leukotriene B_4 binding activity (K_d = 3.67 nM). The apparent low affinity of the expressed BLT1 is thought to be due to relative non-availability of the $G\alpha_i$ isoform, which couples to BLT1, in BV. Co-infection of heterotrimeric G protein recombinant viruses led to co-expression of BLT1 and G protein subunits on BV. A guanosine-5′-(β,γ-imido)triphosphate-sensitive, high affinity ligand binding was observed in the BLT1 BV co-expressing $G\alpha_{i1}\beta_1\gamma_2$ (K_d = 0.17 nM). A relatively large amount of high affinity receptor protein was recovered in the co-expressing BV fraction (6.81 pmol/mg of protein). A combination of BLT1 and $G\alpha_{i1}$ without $G\beta_1\gamma_2$ did not exhibit high affinity ligand binding on BV, indicating the low background environment for the GPCR-G protein coupling in this BV reconstitution system. To test other G proteins for coupling, various Gα subunits were combinatorially expressed in BV with BLT1 and $G\beta_1\gamma_2$. The BLT1 BV co-expressing $G\alpha_{oA}\beta_1\gamma_2$ exhibited a comparably high affinity ligand binding as well as ligand-stimulated guanosine 5′-3-*O*-(thio)triphosphate binding to $G\alpha_{i1}\beta_1\gamma_2$. Co-expression of other Gα isoforms such as $G\alpha_s$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$, $G\alpha_{12}$, or $G\alpha_{13}$ did not exhibit any significant effects on ligand binding affinity in this system. These results reveal that BLT1 and coupled trimeric G proteins were functionally reconstituted on BV and that $G\alpha_o$ as well as $G\alpha_i$ couples to BLT1. This expression system should prove highly useful for pharmacological characterization, biosensor chip applications, and also drug discovery directed at highly important targets of the membrane receptor proteins.

G protein-coupled receptors (GPCRs)[1] comprise a superfamily of membrane proteins made up of seven transmembrane segments. GPCRs play an important role in tranamembrane signal transduction by binding extracellular ligands such as biogenic amines, peptides, hormones, lipids, and nucleotides (1). The sensing of exogenous stimuli such as light, odors, and taste is also mediated by this family of GPCRs. Ligand binding activates the heterotrimeric guanine nucleotide-binding proteins (G proteins) on the intracellular face of the plasma membrane (2–5). There is a considerable interest in the investigation of newly identified GPCRs as potential therapeutic targets (6–8).

The expression of GPCRs in insect cells with recombinant baculovirus is a common technique (9). Sf9 cells, derived from *Spodoptera frugiperda*, are essentially free of endogenous GPCRs (10), providing a low background environment for ligand binding assays. Sf9 cells do endogenously contain certain classes of G proteins that couple to heterologously expressed GPCRs. However, various lines of evidence have made it clear the endogenous G_i-class G proteins exist only at low levels in Sf9 cells (11, 12). The 5-hydroxytryptamine (10, 13–15), M_2 muscarinic (16), D_2 dopamine (17), α_{2A} adrenergic (18), and formylmethionylleucylphenylalanine (11) receptors have been shown to increase ligand binding activity and/or GTP binding

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[1] The abbreviations used are: GPCR, guanine nucleotide-binding protein (G protein)-coupled receptor; Gα, G protein α-subunit; $G\beta_1\gamma_2$, G protein $\beta_1\gamma_2$ dimer; K_d, agonist dissociation constant; K_i, agonist inhibition constant; B_{max}, relative maximum binding; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; Sf9, *S. frugiperda* 9; LTB_4, leukotriene B_4 (5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid); BLT1, leukotriene B_4 receptor type 1; His-BLT1, His-tagged BLT1; BV, budded virus; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.

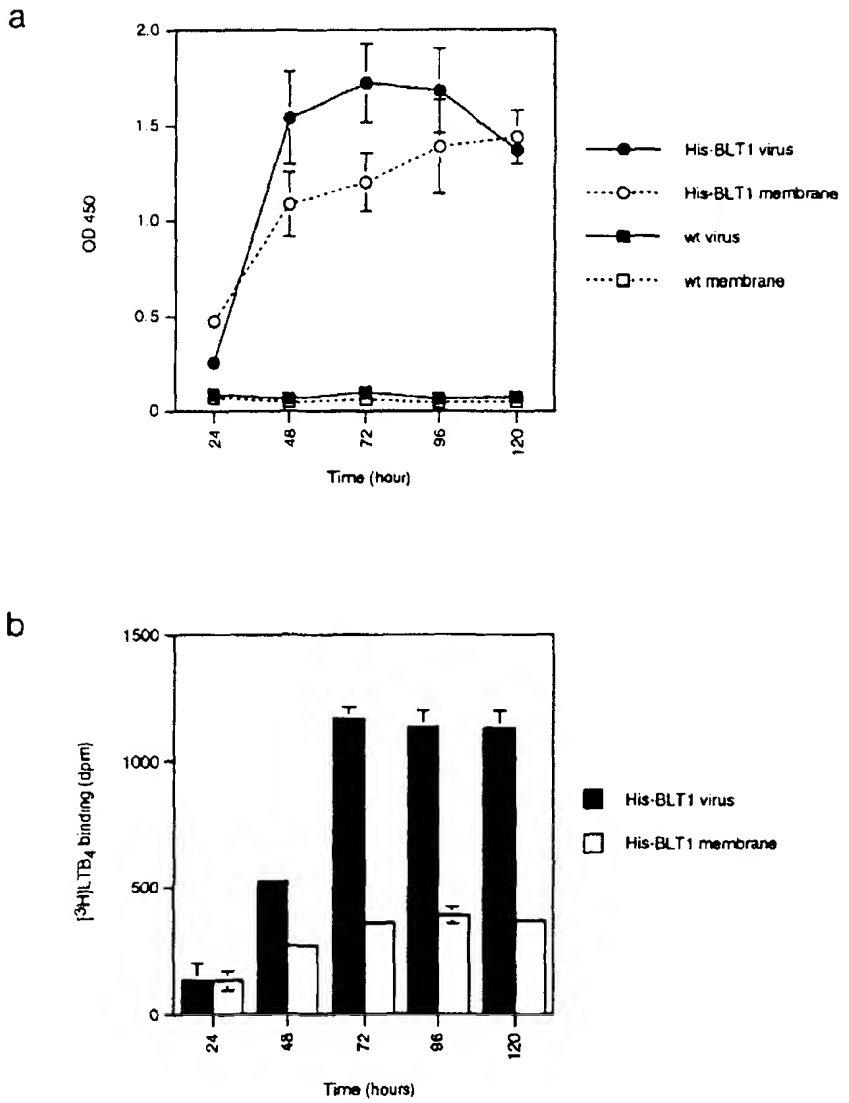

FIG. 1. **Time course of BLT1 expression on budded virus and Sf9 cells.** The expression of His-tagged BLT1 was detected by ELISA with anti-His antibody (*a*) or by [^{3}H]LTB_4 binding (*b*). The BV and membrane fractions were collected at various times after infection. *a*, the BV fractions were collected from Sf9 cells infected with His-BLT1 recombinant (●) or wild type (*wt*, ■) baculovirus. The membrane fractions were also prepared from Sf9 cells infected with His-BLT1 recombinant (○) or wild type virus (□). Samples (5 μg of protein) were used to detect the immunoreactivity. *b*, 1 μg of protein of the BV or membrane fractions was incubated with 0.25 nM [^{3}H]LTB_4, and bound radioactivity was detected. Data represent the mean ± S.D. (n = 4).

activity in Sf9 cells when co-expressed with G_i-class G proteins.

Although Sf9 cells are able to support very high expression levels of exogenous genes (19), the D_2 dopamine receptor expressed in Sf9 cells is largely unglycosylated (20). In the case of the β_2-adrenergic receptor and the formylmethionylleucylphenylalanine receptor, immature and incompletely glycosylated forms of these receptors in the cell membrane fraction of Sf9 cells have also been reported (11, 21, 22).

Loisel *et al.* (23) successfully recovered functional β_2-adrenergic receptor from extracellular baculovirus particles by co-infection of β_2-adrenergic receptor recombinant baculovirus with a baculovirus encoding the Pr55 Gag product of the human immunodeficiency virus type 1. In contrast with what had been expected, most of the recombinant β_2-adrenergic receptor was excluded from the Gag particles and were found instead to be associated with extracellular baculovirus (BV) in a budded form. Surprisingly, the β_2-adrenergic receptor expressed on the virus particles had been glycosylated and was biologically active. In addition, post-translational modifications were found upon agonist stimulation. It should be noted here that β_2-adrenergic receptor is a G_s-coupling receptor (24), and Sf9 cells contain the G_s class G protein in some abundance (12).

BLT1 is known to be the high affinity receptor for leukotriene B_4 (LTB_4), which is found mainly in leukocytes and has been implicated in inflammatory activity (25–28). BLT1 is also reported to couple to G_i- and G_q-class G proteins (29, 30) and is not involved in G_s-class G protein signaling. In a previous report, guinea pig brain membrane exhibited LTB_4-specific binding (31). It is also reported that LTB_4 binding to partially purified membrane fraction from porcine leukocytes was in-

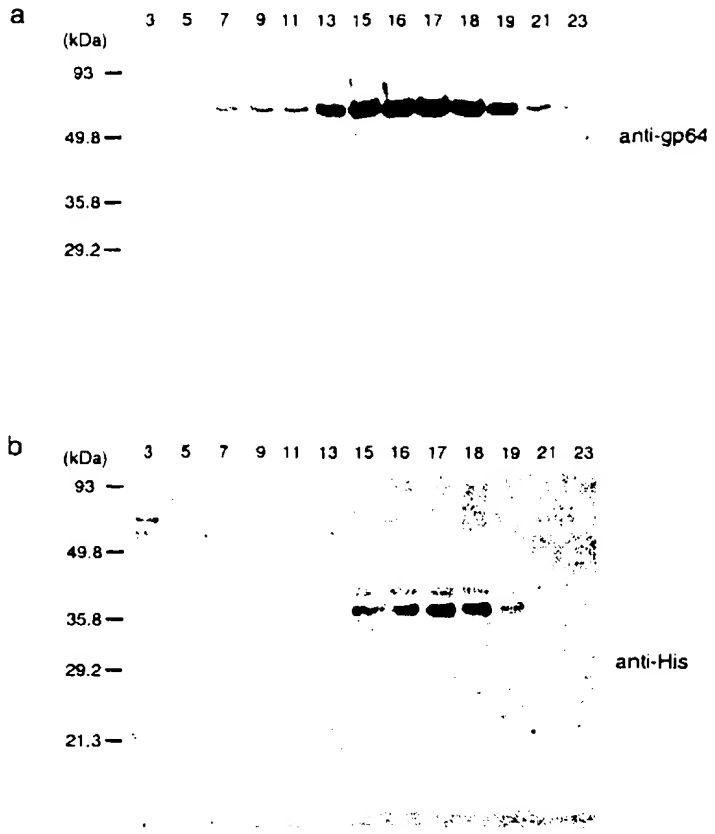

FIG. 2. **Density gradient centrifugation of BV expressing BLT1.** The BV fraction collected from the culture medium of Sf9 cells infected with His-tagged BLT1 recombinant virus was fractionated by sucrose density gradient sedimentation as described under "Experimental Procedures." Each 10-μl fraction (indicated on the *top* of the *panels*) was separated by 12% SDS-PAGE. The blotted membranes were immuno-stained with anti-gp64 (*panel a*) or anti-His (*panel b*) antibodies. The positions of the molecular mass marker proteins are indicated on the *left*.

TABLE I

Binding of [^{3}H]LTB$_4$ to BLT1 expressed on membrane and BV fractions

Ligand binding experiments for various Sf9 membrane or BV fractions expressing receptor and G proteins were performed as described under "Experimental Procedures." K_d and B_{max} values were derived from the best fit curve to a two-binding-site model using non-linear analysis. ND, not determined.

Coexpressed proteins	High affinity binding		Low affinity binding	
	K_d (mean ± S.E.)	B_{max} (mean ± S.E.)	K_d (mean ± S.E.)	B_{max} (mean ± S.E.)
	nM	*pmol/mg of protein*	*nM*	*pmol/mg of protein*
Membrane fraction				
BLT1	0.218 ± 0.061	1.372 ± 0.456	4.378 ± 0.103	26.53 ± 5.772
BLT1 $G\alpha_{i1}$	0.099 ± 0.004	3.781 ± 0.066	5.834 ± 0.149	12.28 ± 0.417
BLT1 $G\beta_1\gamma_2$	0.202 ± 0.027	2.082 ± 0.161	5.540 ± 1.290	2.986 ± 0.828
BLT1 $G\alpha_{i1}\beta_1\gamma_2$	0.114 ± 0.014	4.887 ± 0.155	ND	ND
BV fraction				
BLT1	0.126 ± 0.051	0.638 ± 0.130	3.670 ± 0.061	27.34 ± 0.550
BLT1 $G\alpha_{i1}$	0.147 ± 0.027	1.921 ± 0.176	2.997 ± 0.128	13.01 ± 0.623
BLT1 $G\beta_1\gamma_2$	0.138 ± 0.030	0.567 ± 0.061	3.632 ± 0.113	6.481 ± 0.252
BLT1 $G\alpha_{i1}\beta_1\gamma_2$	0.171 ± 0.014	6.814 ± 0.164	ND	ND

creased by reconstitution of G_i-class G proteins including $G\alpha_o$ (32), which is found in the brain and nervous system (33). Although these reports suggest that $G\alpha_o$ mediates LTB_4 signaling, the coupling of $G\alpha_o$ with BLT1 has not been investigated intensively. To exploit extracellular baculovirus expression to test whether GPCR and G protein could be reconstituted on the BV, we studied the expression of human BLT1. The possible selectivity of BLT1 coupling with other classes of G proteins was also studied by means of this system.

EXPERIMENTAL PROCEDURES

Reagents—LTB_4 was purchased from Cayman Chemical Co. (Ann Arbor, MI). [^{3}H]LTB_4 (6016.2 GBq/mmol) and [^{35}S]GTPγS (46.2 TBq/mmol) were purchased from PerkinElmer Life Sciences. The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Other chemicals or reagents were purchased from Sigma unless otherwise noted. A recombinant baculovirus expression vector pAcYMI (34) was kindly provided by Dr. Y. Matsuura (the Research Institute for Microbial Disease, Osaka University, Japan). A baculovirus-expressing bovine $G\gamma_2$ (35) was generously provided by Dr. T. Kozasa (the Department of Pharmacology, University of Illinois at Chicago). The cDNA clones for human $G\alpha_{oA}$, $G\alpha_{12}$, $G\alpha_{14}$, and $G\alpha_{16}$ were provided by the Guthrie cDNA Resource Center (www.cdna.org).

Recombinant Baculovirus Construction and Sf9 Cell Culture—The cDNA for human BLT1 (29) was amplified by PCR using a 5′ sense primer containing the *Bam*HI site and a 3′ antisense primer containing the *Eco*RI site and inserted into pBlueBac4.5 and pBlueBacHis2A bacu-

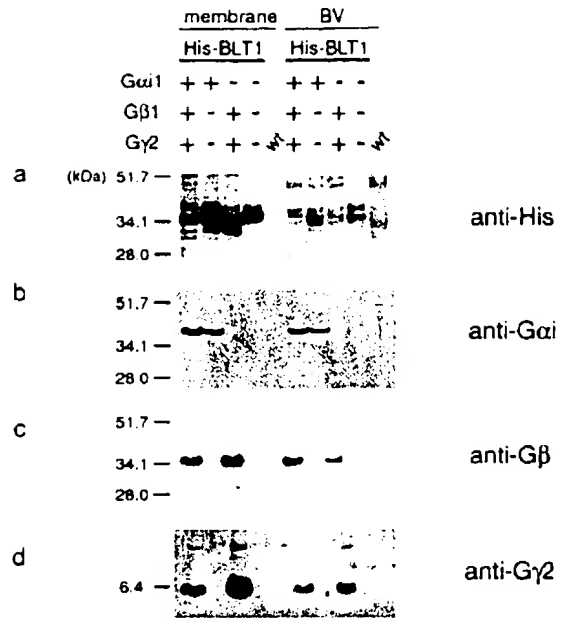

FIG. 3. **Western blots of Sf9 cell membrane and BV fractions expressing receptor and G protein subunits.** Sf9 cells were co-infected with various combinations of recombinant baculoviruses encoding His-BLT1, $G\alpha_{i1}$, $G\beta_1$, and $G\gamma_2$, as indicated on the *top* of *panel*. The membrane fraction and BV fraction were prepared from infected cells and culture supernatants, respectively. Because of the difference of expression level of BLT1 in each sample the membrane or BV fraction was loaded in *panel a* as follows: the membrane fraction expressing His-BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ (30 μg), His-BLT1 and $G\alpha_{i1}$ (30 μg), His-BLT1 and $G\beta_1\gamma_2$ (30 μg), His-BLT1 alone (3 μg) from cells infected with wild type (*wt*) baculovirus (30 μg); the BV fraction expressing His-BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ (50 μg), His-BLT1 and $G\alpha_{i1}$ (50 μg), His-BLT1 and $G\beta_1\gamma_2$ (50 μg), His-BLT1 alone (5 μg), and wild type baculovirus (50 μg). In each lane of *panel b–d*, 30 μg of the membrane or BV fraction was loaded. The blotted membrane was immuno-stained with either anti-His (*panel a*), anti-$G\alpha_i$ (*panel b*), anti-$G\beta$ (*panel c*), or anti-$G\gamma_2$ (*panel d*) antibody. The positions of the molecular mass marker proteins are indicated on the *left*.

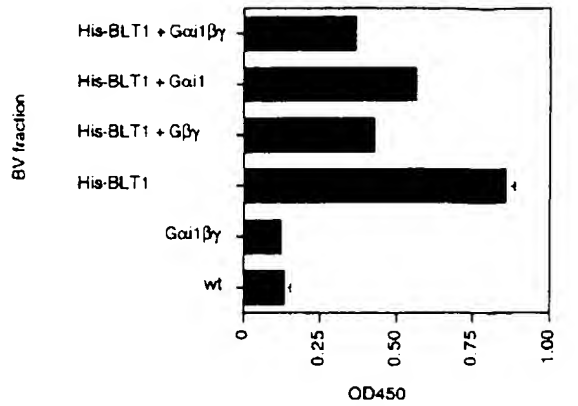

FIG. 4. **Expression levels of BLT1 on BV co-expressing G protein subunits.** BV samples expressing His-tagged BLT1 with or without various G protein subunits were subjected to ELISA with an anti-His antibody (n = 4, mean ± S.D.). Samples of 3 μg of protein were used. $\beta\gamma$ and wild type (*wt*) represent the BV expressing $G\beta_1$ plus $G\gamma_2$ subunits and the budded wild type baculovirus, respectively. *OD*, optical density.

lovirus expression vectors (Invitrogen). These were designated as pBB4.5-BLT1 and pBBH2A-BLT1, respectively. For pBBH2A-BLT1, the residual coding sequence between the enterokinase restriction site of the vector and the first Met of BLT1 was deleted by site-directed mutagenesis using the primers 5′-CTGTACGACGATGACGATAAGAT-GAACACTACATCTTCTGCAGC-3′ and 5′-GCTGCAGAAGATGTAGT-GTTCATCTTATCGTCATCGTCGTACAG-3′, and the resulting plasmid was designated pBBH2-BLT1. Rat $G\alpha_o$ cDNA (36) was digested with *Nco*I and *Hin*dIII and inserted into pBlueBacIII (Invitrogen). Rat $G\alpha_{i1}$ cDNA (37) mutated to lack an endogenous *Nco*I site in the coding region was digested with *Nco*I and *Hin*dIII and inserted into pBlueBacIII. Mouse $G\alpha_{i1}$ cDNA (38) was digested with *Hin*dIII and inserted into pBlueBacIII. Rat $G\alpha_{i2}$ cDNA (39) was digested with *Bsu*36I and *Bgl*II and treated with Klenow fragment to generate blunt ends. Baculovirus expression vector pAcYMI was digested with *Bam*HI and treated with Klenow fragment. The blunt-end fragments of the vector were ligated with $G\alpha_{i2}$ cDNA. Bovine $G\beta_1$ cDNA was amplified by PCR using a 5′ sense primer containing the *Sma*I and *Xho*I sites and a 3′ antisense primer containing the *Sma*I and *Eco*RI sites and inserted into pAcYMI at the *Sma*I site. The cDNAs for human $G\alpha_{oA}$, $G\alpha_{i3}$, $G\alpha_{i4}$, or $G\alpha_{i5}$ were subcloned individually into pBlueBac4.5 at the *Kpn*I and *Xba*I sites. The nucleotide sequences were verified by DNA sequencing. To generate recombinant viruses, Sf9 cells were co-transfected with the vector for either the BLT1, $G\alpha_{oA}$, $G\alpha_{i3}$, $G\alpha_{i4}$, or $G\alpha_{i5}$ and Bac-N-Blue viral DNA (Invitrogen). Other vectors for the G protein subunits were co-transfected individually with BaculoGold viral DNA (BD Biosciences). All viruses were produced by homologous recombination in Sf9 cells. The recombinant baculoviruses thus obtained were plaque-purified and amplified. Sf9 cells were cultured in Grace's supplemented media (Invitrogen) containing 10% fetal calf serum, 0.1% Pluronic F-68 (Invitrogen), 100 international unit/ml penicillin and 100 μg/ml streptomycin in a 1-liter spinner flask at 27 °C. Cells (2×10^6 cells/ml) were infected with the recombinant baculovirus at a multiplicity of infection of 5 for 72–120 h. For production of the BV of baculovirus co-expressing receptor and G proteins, Sf9 cells (2×10^6 cells/ml) were co-infected with recombinant baculoviruses at a multiplicity of infection of 2 for each virus and harvested 72 h after infection.

Viral Particle Preparation—The BV of baculovirus was isolated basically according to the method of Loisel *et al.* (23). All procedures were carried out at 0–4 °C unless stated otherwise. The culture medium of Sf9 cells infected with recombinant baculovirus was centrifuged at 1,000 × *g* for 15 min to separate cells. Then the supernatant was centrifuged at 40,000 × *g* for 30 min. The pellet was resuspended in phosphate-buffered saline and centrifuged at 1,000 × *g* for 15 min to eliminate possible contamination with cell debris. The supernatant was again centrifuged at 40,000 × *g* for 30 min. The precipitates, used as BV fractions, were resuspended in phosphate-buffered saline or assay buffer and stored at 4 °C until use.

For preparation of the membrane fraction, the cells were resuspended in HE/PI buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) (40). Cells were broken open by a path made with a 26-gauge needle inserted 15 times. The homogenates were centrifuged at 1,000 × *g* for 5 min, and the supernatants were further centrifuged at 20,000 × *g* for 30 min. The precipitates (the membrane fractions) were suspended in HE/PI buffer and stored at −20 °C until use.

Sucrose Density Gradient Sedimentation—Fractionation of the BV fraction by sucrose density gradient sedimentation was performed as described by Loisel *et al.* (23). Briefly, 600 μg of the BV fraction, which had been suspended in 1.2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was layered onto 36 ml of a linear 25–56% (w/v) sucrose gradient in TE buffer. The sample was centrifuged at 10,000 × *g* for 90 min. Fractions of 1.5 ml were collected from the top of the tube. Then each fraction was centrifuged at 20,000 × *g* for 45 min. The precipitates were resuspended with phosphate-buffered saline and used for Western blot analysis.

Electrophoresis and Immunoblotting—SDS-PAGE was carried out as described by Laemmli (41), except that samples were not heat-treated so as to minimize aggregation. For immunoblotting, proteins expressed on BV were resolved by SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences) electrophoretically at 20 V overnight. Membranes were incubated for 1 h with Block Ace (Snow Brand Milk Products, Japan) at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibody in Tris-buffered saline (TBS; 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.4). The following antibodies were used: monoclonal mouse anti-baculoviral glycoprotein gp64 (established in our laboratory); monoclonal mouse anti-polyhistidine (Sigma); polyclonal rabbit anti-$G\alpha_o$ (K-20 sc-823), anti-$G\alpha_{oA}$ (K-20 sc-387), anti-$G\alpha_{i1}$ (D-17 sc-394), anti-$G\alpha_{i2}$ (S-20 sc-409), anti-$G\beta$ (T-20 sc-378), anti-$G\gamma_2$ (A-16 sc-374), and polyclonal

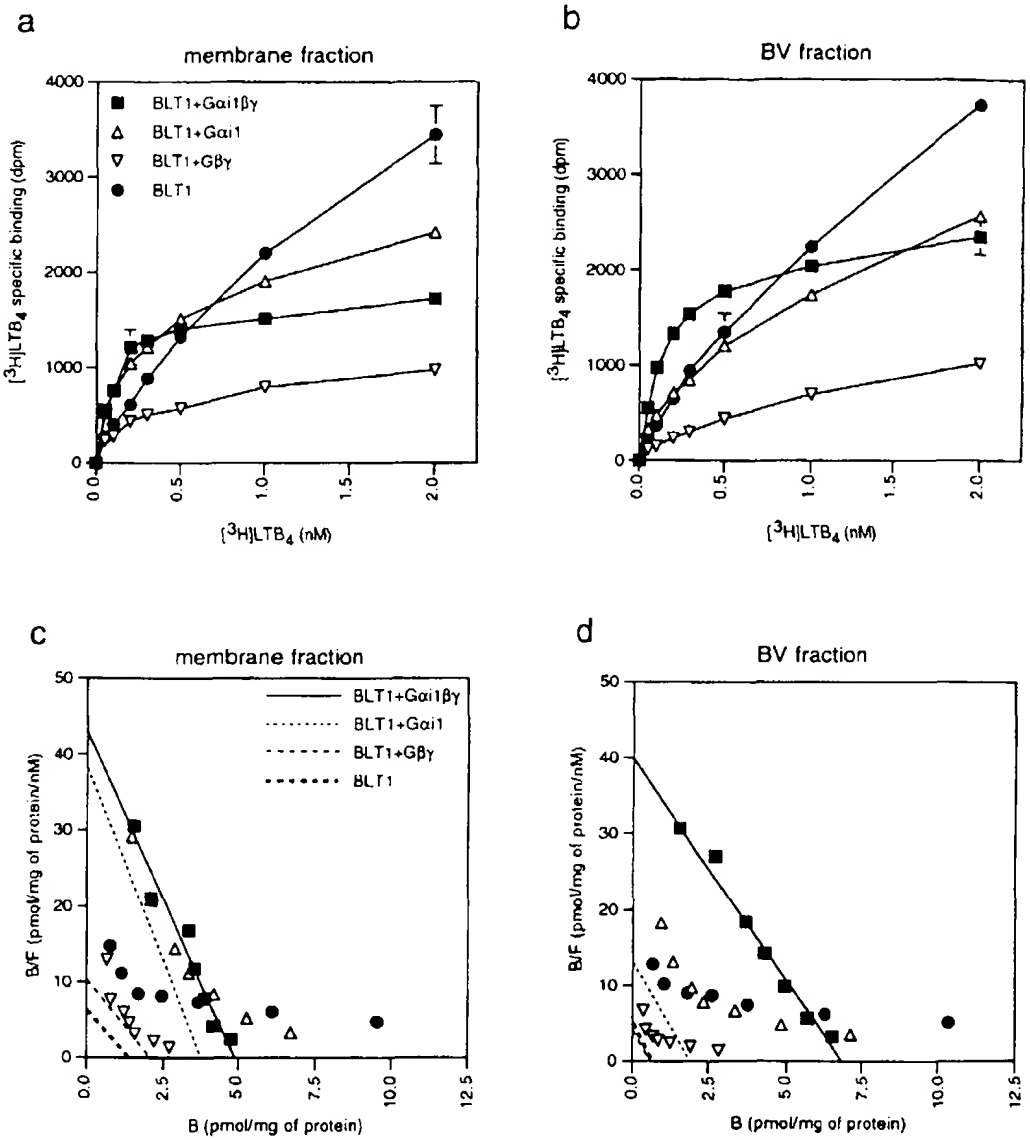

FIG. 5. **[^{3}H]LTB$_4$ binding to the membrane or BV fractions from Sf9 cells co-infected with recombinant baculoviruses encoding BLT1 and G protein subunits.** Binding isotherms and Scatchard analyses of various membrane or BV fractions co-expressing BLT1 and G protein subunits are shown. Values are represented as the means ± S.E. (n = 4). The membrane or BV fractions (1 μg each) expressing BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ (■), BLT1 and $G\alpha_{i1}$ (△), BLT1 and $G\beta_1\gamma_2$ (▽), and BLT1 alone (●) were analyzed. Isotherms and Scatchard analyses were best fitted to a two-binding-site model using nonlinear analysis. In the Scatchard plot, only the high affinity binding sites from each membrane or BV fractions are designated as indicated on the figure panels for BLT1 and $G\alpha_{i1}\beta_1\gamma_2$, BLT1 and $G\alpha_{i1}$, BLT1 and $G\beta_1\gamma_2$, and BLT1 alone. The K_d and B_{max} values were derived from each individual experiment, and the means ± S.E. are summarized in Table I.

goat anti-$G\alpha_{16}$ (C-18 sc-7415) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal rabbit anti-$G\alpha_i$ (AS/7, PerkinElmer Life Sciences); anti-$G\alpha_{13}$ (3A-190, Gramsch Laboratories, Schwabhausen, Germany); anti-$G\alpha_{14}$ (3A-196, Gramsch Laboratories). Membranes were washed 4 times with TBS with 0.05% Tween 20 and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG as the secondary antibody. Membranes were then washed 3 times with TBS with 0.05% Tween 20 and incubated with SuperSignal West Dura substrate (Pierce). Membranes were then exposed to x-ray film RX-U (Fuji Photo Film, Tokyo, Japan).

Enzyme-linked Immunosorbent Assay—The BV or membrane fractions were adsorbed to the wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Asahi Techno Glass, Tokyo, Japan) overnight at 4 °C. Wells were washed and blocked with TBS, pH 7.4, containing 40% Block Ace for 1 h at room temperature. After incubation with mouse monoclonal anti-polyhistidine antibody for at least 1 h, wells were washed 3 times with TBS with 0.05% Tween 20. The anti-polyhistidine antibody bound to wells was detected with the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody followed by the addition of substrate (tetramethylbenzidine). The reaction was terminated by acid, and absorbances at 450 nm were quantitated using a 96-well plate reader.

Ligand Binding Assay—The radiolabeled ligand binding assay was carried out in a total of 200 μl of binding buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM NaCl, 0.1% fatty acid-free bovine serum albumin) (29, 42) containing the BV fraction and [^{3}H]LTB$_4$. To test the effect of Gpp(NH)p on [^{3}H]LTB$_4$ binding to the BV fractions, 0.02% (w/v) of saponin was added in the binding buffer with various amounts of Gpp(NH)p. The assay mixtures were incubated at room temperature for 1 h. The incubation was terminated by filtration of the assay mix-

tures through GF/C glass fiber filters (PerkinElmer Life Sciences). The filters were washed with ~2 ml of ice-cold binding buffer and dried, and the remaining radioactivity was measured in a Top-count microplate scintillation counter (PerkinElmer Life Sciences). The data were analyzed with the program GraphPad Prism (GraphPad Software, San Diego, CA).

Receptor-promoted [^{35}S]GTPγS Binding Assay—The BV fractions (3 μg each) were resuspended in assay buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) (43) supplemented with 0.1 mM dithiothreitol, 0.1% (w/v) fatty acid-free bovine serum albumin, and 0.02% (w/v) saponin. BV fractions were incubated with GDP and 0.3 nM [^{35}S]GTPγS with or without 100 nM LTB_4 at room temperature for 1 h. The incubation was terminated by rapid filtration through GF/C filters (PerkinElmer Life Sciences). The filters were then washed with ~2 ml of ice-cold assay buffer and dried, and the remaining radioactivity was measured in a Top-count microplate scintillation counter (PerkinElmer Life Sciences).

RESULTS

Expression of BLT1 on Budded Baculovirus

The BV fraction was recovered from the culture medium of Sf9 cells infected with His-tagged BLT1 (His-BLT1) recombinant baculovirus. BLT1 expression, detected by ELISA with anti-His antibody, was seen in the BV fraction 48 h after infection with recombinant virus carrying His-BLT1 cDNA (Fig. 1*a*). The His-BLT1 expression reached its peak 72 h after infection. The amount of His-BLT1 in the BV fraction at 72 h was 1.4-fold higher than that of the membrane fraction from His-BLT1-infected Sf9 cells. [^{3}H]LTB_4 binding activity was also observed in both the BV and membrane fractions prepared from Sf9 cells that had been infected with His-BLT1 recombinant baculovirus (Fig. 1*b*). The maximum binding activity was reached at 72 h after infection, which was about 3-fold higher in the BV than the membrane fraction. No binding activity was detected in the BV or membrane fraction collected from Sf9 cells infected with wild type baculovirus.

To clarify whether BLT1 is expressed on virus or cell debris, the BV fraction from the culture medium of Sf9 cells infected with BLT1 recombinant baculovirus was analyzed by ultracentrifugation in sucrose density gradients. As shown in Fig. 2, His-BLT1 was found to cosediment with baculovirus glycoprotein gp64. The expression time course and properties of the sedimentation of BLT1 in culture supernatant are compatible with previously reported β_2-adrenergic receptor expression on extracellular budded virus (23). Thus, BLT1 is likewise deduced in this case to be expressed on the budded baculovirus. The affinity for the [^{3}H]LTB_4 of BLT1 expressed on BV seems to be low (K_d = 3.67 nM, Table I) compared with that of the membrane fraction of Cos-7 cells expressing BLT1 (K_d = 0.17 nM) (29). This is considered to be due to the low level of the G_i-class G protein in Sf9 cells, which is needed to couple to BLT1 for high affinity binding.

Reconstitution of BLT1-G Protein Coupling on Budded Virus

Expression of Receptor and Heterotrimeric G Protein—To check for BLT1-G protein coupling on BV, we prepared recombinant baculoviruses containing the cDNAs encoding the His-BLT1 as well as the G protein α_{i1}, β_1, and γ_2 subunits. His-BLT1 and the G protein subunits $G\alpha_{i1}$, $G\beta_1$, and $G\gamma_2$ were expressed on BV in various combinations. After 72 h of infection, each BV fraction was collected. The amount of expressed receptor or each subunit of G protein was assessed by Western blotting (Fig. 3). When the sample was heat-treated for SDS-PAGE, BLT1 stacked in the stacking gel and did not enter the separating gel. Therefore, we performed SDS-PAGE analysis without heat treatment. For BLT1, the deduced molecular mass from the amino acid sequence is 37.6 kDa. Using anti-His antibody immunoblotting, three bands, with apparent molecular masses of 36.6, 35.5, and 34.8 kDa, were detected in His-BLT1-expressing BV. We did not observed the bands corresponding to the dimmers of the BLT1 in this system even in the presence of G protein. The amount of receptor expression decreased with co-infection of various G protein subunits in both the membrane and BV fractions (Fig. 3*a*). This observation was corroborated with ELISA measurement using an anti-His antibody (Fig. 4). No immunoreactive band was detected with anti-His antibody in the wild type BV sample. Each G protein subunit expressed an apparently similar amount on BV.

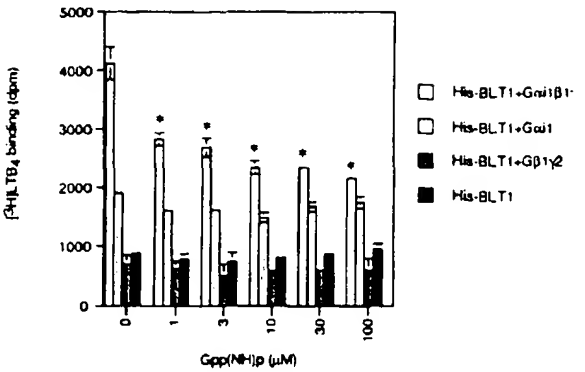

FIG. 6. **The effect of Gpp(NH)p on ligand binding to BLT1 co-expressed with G protein on BV.** Specific binding of [^{3}H]LTB_4 (added at a concentration of 0.25 nM) to 3 μg of BV (n = 4, mean ± S.D.). Budded virus expressing BLT1 and $G\alpha_{i1}\beta_1\gamma_2$, BLT1 and $G\alpha_{i1}$, BLT1 and $G\beta_1\gamma_2$, and BLT1 alone (as indicated in the figure) were analyzed. Statistical analysis was performed using Student's t test. Significant inhibition by Gpp(NH)p is indicated as $p < 0.001$ (*).

Ligand Binding Assay of Reconstituted Receptor and G Protein—The BV fractions recovered from culture medium 72 h after infection with His-BLT1 recombinant virus displayed a dose-dependent binding for [^{3}H]LTB_4 (Fig. 5, *a* and *b*). In Scatchard plot analysis, the binding properties of [^{3}H]LTB_4 to BLT1 expressed on BV or membrane fractions contain both high and low affinity sites (Fig. 5, *c* and *d*). The K_d and B_{max} values calculated from the Scatchard plot are shown in Table I. As shown in Table I, the ligand binding properties were similar in the Sf9 membrane and BV fractions expressing the receptor alone. For BV-expressed BLT1, the mean K_d and B_{max} values of the high affinity binding sites were 0.13 nM and 0.64 pmol/mg of protein, and those of the low affinity sites were 3.67 nM and 27.3 pmol/mg of protein, respectively (n = 4). The His hexapeptide fused to the N terminus of the BLT1 did not affect ligand binding (data not shown). There was no significant specific [^{3}H]LTB_4 binding in the BV fraction prepared from the culture medium of Sf9 cells infected with the wild type baculovirus.

To estimate the effects of G protein on agonist affinity, the Sf9 membrane and BV fractions co-expressing receptor and G proteins were used for the [^{3}H]LTB_4 binding experiments (Fig. 5). As shown in Table I, the B_{max} value of the membrane fraction co-expressing with BLT1 and $G\alpha_{i1}$ was 3.78 pmol/mg of protein, which is 2.8-fold greater than the membrane fraction expressing BLT1 alone. The co-expression of $G\beta_1\gamma_2$ subunits with BLT1 also increased the high affinity site by 1.5-fold in the membrane fraction. For the BV fraction, the sample co-expressing BLT1 and $G\alpha_{i1}$ exhibited a relatively small number of high affinity binding sites (B_{max} = 1.92 pmol/mg of protein). Reconstitution of the G protein heterotrimer ($G\alpha_{i1}\beta_1\gamma_2$) switched almost all the entire receptor population to the high affinity state with a K_d value of 0.17 nM, increasing the B_{max} value of the high affinity receptor 9.5-fold compared with the receptor expressed alone.

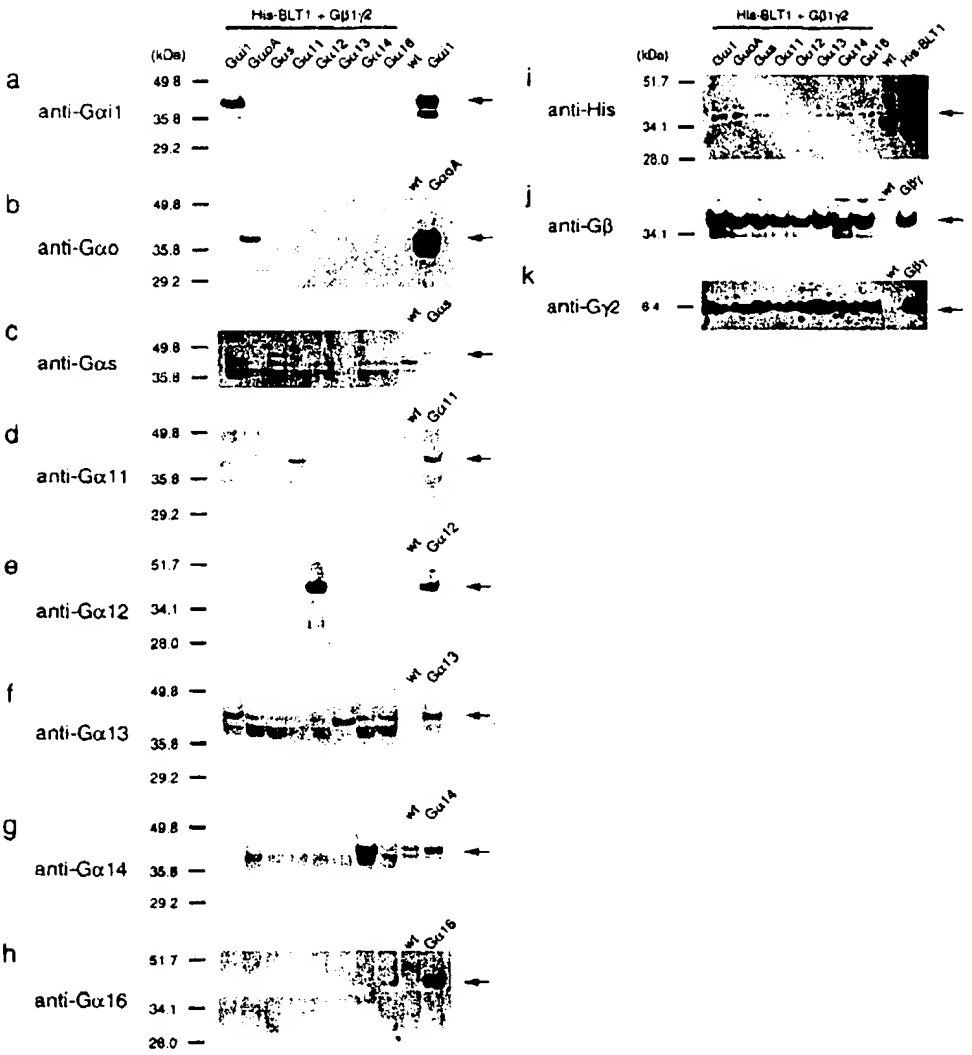

FIG. 7. **Western blot of BV fractions co-expressing BLT1 and various Gα subunits with $G\beta_1\gamma_2$.** The BV fractions were collected from the culture supernatant of Sf9 cells co-infected with various combinations of recombinant viruses encoding His-BLT1 as well as $G\alpha_{i1}$, $G\alpha_{oA}$, $G\alpha_o$, $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{14}$, or $G\alpha_{16}$ in addition to His-BLT1 and $G\beta_1\gamma_2$ as shown on the *top* of *panel*. On each lane, 30 μg of BV fraction was loaded. In each panel, BV expressing only G protein subunit or His-BLT1 was loaded in the *right end panel*. Immuno-staining was performed with an anti-$G\alpha_i$ (*panel a*), an anti-$G\alpha_o$ (*panel b*), an anti-$G\alpha_s$ (*panel c*), an anti-$G\alpha_{11}$ (*panel d*), an anti-$G\alpha_{12}$ (*panel e*), an anti-$G\alpha_{13}$ (*panel f*), an anti-$G\alpha_{14}$ (*panel g*), an anti-$G\alpha_{16}$ (*panel h*), an anti-His antibody (*panel i*), an anti-Gβ (*panel j*), and an anti-$G\gamma_2$ (*panel k*) antibody. The *arrows* indicate the specific band recognized by each antibody. The positions of the molecular mass marker proteins are indicated on the *left*. *wt*, wild type.

The Effect of Gpp(NH)p on Ligand Binding to BV Expressing BLT1 and G Proteins

A GTP analogue, Gpp(NH)p, which is not hydrolyzed by the GTPase activity of the Gα protein, was used to delineate receptor-G protein coupling. Gpp(NH)p decreased [^{3}H]LTB_4 binding to BV expressing His-BLT1 and $G\alpha_{i_2}\beta_1\gamma_2$, providing evidence for the receptor-G protein coupling (Fig. 6). On the other hand, the BLT1 BV, BLT1 with $G\alpha_{i1}$ BV, and BLT1 with $G\beta_1\gamma_2$ BV were not significantly affected in ligand binding by the addition of Gpp(NH)p. These results suggest that the co-expression of the receptor and heterotrimeric G protein reconstitute to functional GPCR-G protein complex on BV.

The Effect of the Co-expression of Various Classes of G Protein on the [^{3}H]Leukotriene B_4 Binding Properties of BLT1 Expressed on BV

To compare the effect of the G protein α subunit of various classes on ligand binding, Sf9 cells were co-infected with various combinations of recombinant baculovirus encoding several different classes of G protein subunits (α with $G\beta_1\gamma_2$ in every case) and His-BLT1. Each BV fraction was collected by centrifugation, and each of the expressed proteins was detected by Western blotting (Fig. 7). The expression levels of His-BLT1 assessed by ELISA were similar among the BV fractions expressing different G proteins (data not shown). No specific

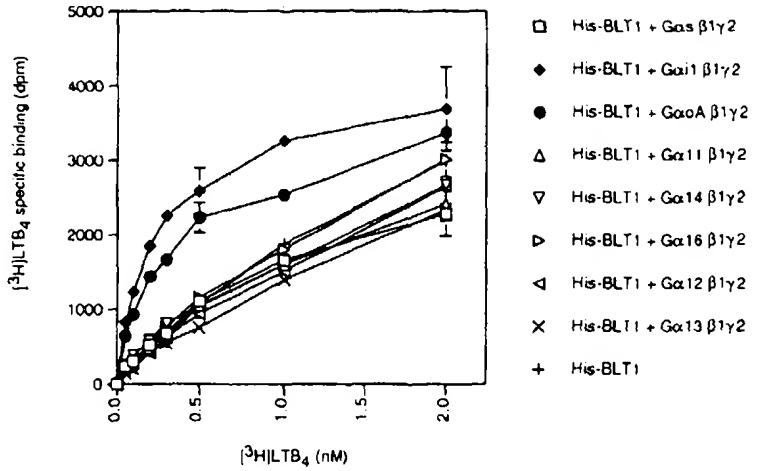

FIG. 8. **[^{3}H]LTB$_4$ binding to the BV fractions co-expressing BLT1 and various classes of Gα with Gβγ.** Binding isotherms of BV co-expressing BLT1 and various classes of Gα subunits with $G\beta_1\gamma_2$ are shown (n = 4, mean ± S.E.). [^{3}H]LTB$_4$ binding to budded viruses expressing BLT1 and $G\alpha_s\beta_1\gamma_2$ (□), BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ (◆), BLT1 and $G\alpha_{oA}\beta_1\gamma_2$ (●), BLT1 and $G\alpha_{11}\beta_1\gamma_2$ (△), BLT1 and $G\alpha_{14}\beta_1\gamma_2$ (▽), BLT1 and $G\alpha_{16}\beta_1\gamma_2$ (▷), BLT1 and $G\alpha_{12}\beta_1\gamma_2$ (◁), BLT1 and $G\alpha_{13}\beta_1\gamma_2$ (×), and BLT1 alone (+) were analyzed. In each assay point, 1 μg of BV expressing BLT1 alone or 2 μg of that of co-expressing BLT1 with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_{oA}\beta_1\gamma_2$ was used. For other BV samples, 3 μg were used. Isotherms were best fitted to a two-binding-site model using nonlinear analysis. The K_d and B_{max} values were derived for each individual experiment, and means ± S.E. are summarized in Table II.

TABLE II
Binding of [^{3}H]LTB$_4$ to BLT1 expressed on BV fractions

Ligand binding experiments for various BV fractions expressing receptor and G proteins were performed as described under "Experimental Procedures." K_d and B_{max} values were derived from the best fit curve to a two-binding-site model using non-linear analysis. ND, not determined.

Coexpressed proteins	High affinity binding		Low affinity binding	
	K_d (mean ± S.E.)	B_{max} (mean ± S.E.)	K_d (mean ± S.E.)	B_{max} (mean ± S.E.)
	nM	*pmol/mg of protein*	*nM*	*pmol/mg of protein*
BLT1 $G\alpha_s\beta_1\gamma_2$	0.174 ± 0.057	0.476 ± 0.083	3.437 ± 0.178	4.716 ± 0.311
BLT1 $G\alpha_{i1}\beta_1\gamma_2$	0.171 ± 0.014	5.139 ± 0.133	ND	ND
BLT1 $G\alpha_{oA}\beta_1\gamma_2$	0.334 ± 0.057	5.141 ± 0.317	ND	ND
BLT1 $G\alpha_{11}\beta_1\gamma_2$	0.144 ± 0.049	0.340 ± 0.057	3.490 ± 0.118	5.278 ± 0.228
BLT1 $G\alpha_{14}\beta_1\gamma_2$	0.109 ± 0.043	0.396 ± 0.064	3.582 ± 0.149	5.587 ± 0.275
BLT1 $G\alpha_{16}\beta_1\gamma_2$	0.121 ± 0.060	0.200 ± 0.047	3.523 ± 0.078	7.055 ± 0.193
BLT1 $G\alpha_{12}\beta_1\gamma_2$	0.199 ± 0.177	0.163 ± 0.078	3.715 ± 0.137	6.374 ± 0.302
BLT1 $G\alpha_{13}\beta_1\gamma_2$	0.165 ± 0.068	0.061 ± 0.023	3.506 ± 0.078	5.720 ± 0.090
BLT1	0.146 ± 0.944	0.038 ± 0.067	3.498 ± 0.058	22.82 ± 0.267

signals were detected by Western blotting in the BV fraction recovered from the culture medium of Sf9 cells infected with wild type baculovirus. The ligand binding properties of these BV fractions were compared. As shown in Fig. 8, an increase of high affinity binding sites was observed in the BV co-infected with His-BLT1 and the G_i heterotrimeric G protein ($G\alpha_{i1}\beta_1\gamma_2$). Furthermore, co-expression of G_o heterotrimeric G protein with His-BLT1 ($G\alpha_{oA}\beta_1\gamma_2$) also led to an increase in the high affinity binding sites. On the other hand, BV fractions co-expressing His-BLT1 with other classes of G protein heterotrimers (G_s, G_{11}, G_{14}, G_{16}, G_{12}, and G_{13}) exhibited a low affinity ligand binding similar to that of BV expressing His-BLT1 alone. As shown in Table II, the B_{max} value of BV co-expressing His-BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_{oA}\beta_1\gamma_2$ in the case of the high affinity sites was similar to that of BV co-expressing other classes of heterotrimeric G proteins in the low affinity sites. These results indicate that almost all the expressed receptors in the former two BV fractions were functionally coupled to $G\alpha_{i1}\beta_1\gamma_2$ and $G\alpha_{oA}\beta_1\gamma_2$.

[^{35}S]GTPγS Binding of Heterotrimeric G Proteins Expressed in Budded Virus

To further examine the selective coupling of G proteins to BLT1, the displacement of [^{35}S]GTPγS binding by GDP in the presence or absence of 100 nM LTB$_4$ was compared among the various BLT1 BV co-expressing different combinations of heterotrimeric G proteins (Fig. 9). Previous studies demonstrate that the agonist increases the apparent affinity of [^{35}S]GTPγS binding sites; it also decreases the affinity of GDP binding sites (44, 45). The binding of [^{35}S]GTPγS was greatly increased by LTB$_4$ in BV co-expressing BLT1 and G_i or G_o heterotrimeric G proteins. Displacement curves for BLT1 BV co-expressed with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_{oA}\beta_1\gamma_2$ were similar to each other both in the presence or absence of the ligand (Fig. 9, *b* and *c*). The IC_{50} values for the GDP of BLT1 BV co-expressing $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_{oA}\beta_1\gamma_2$ were 0.25 and 0.80 μM in the absence of LTB$_4$, and 7.58 and 12.45 μM in the presence of LTB$_4$, respectively. These results show that BLT1 couples to G_o as well as G_i heterotrimeric G proteins in BV.

For the BLT1 BV co-expressing G_s-class G proteins, LTB$_4$ did not exhibit a significant effect on the displacement of [^{35}S]GTPγS binding by GDP (Fig. 9*a*). Although the binding of [^{35}S]GTPγS was apparently induced by LTB$_4$ in BLT1 BV co-expressed with G_q-class heterotrimeric G proteins, the effects of LTB$_4$ were much less than that for BLT1 BV co-expressed with G_i-class G protein (Fig. 9, *d–f*). The GDP IC_{50} values for [^{35}S]GTPγS binding on BLT1 BV co-expressed with G_q-class G proteins ($G\alpha_{11}\beta_1\gamma_2$, $G\alpha_{14}\beta_1\gamma_2$, and $G\alpha_{16}\beta_1\gamma_2$) were increased by 2.3-, 2.4-, and 3.1-fold, respectively, in the presence of LTB$_4$. The BLT1 BV co-expressing $G_{12/13}$-class G protein also showed a slight difference in [^{35}S]GTPγS binding by LTB$_4$ (Fig. 9, *g* and *h*). The IC_{50} values for the GDP of BLT1 BV co-expressed with $G\alpha_{12}\beta_1\gamma_2$ and $G\alpha_{13}\beta_1\gamma_2$ were increased by 2.3- and 2.6-fold, respectively, in the presence of LTB$_4$.

DISCUSSION

Baculovirus infection of Sf9 cells renders the recombinant membrane proteins functionally embedded in the viral parti-

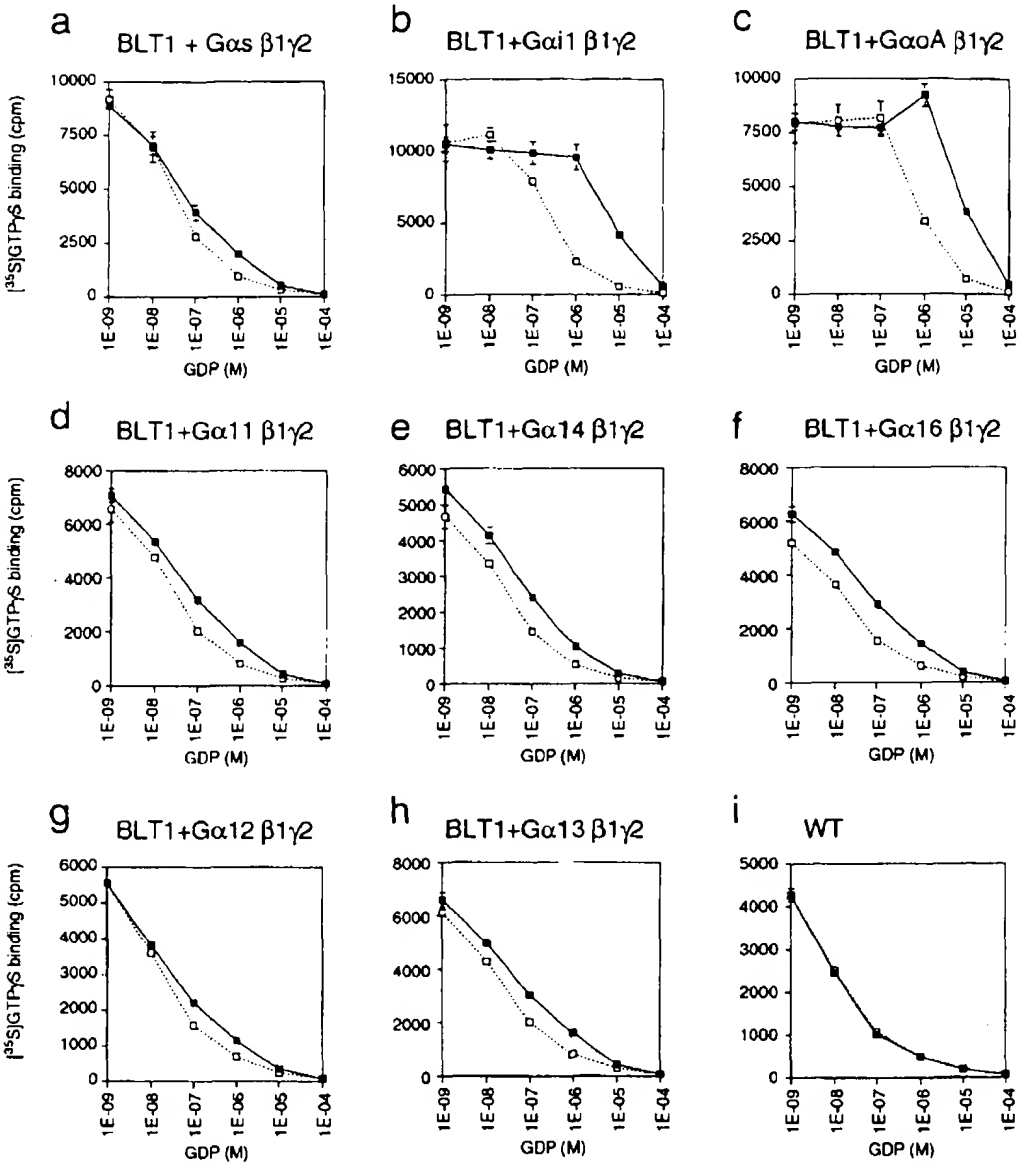

FIG. 9. **Effect of LTB_4 on [^{35}S]GTPγS binding to various classes of Gα subunit expressed on BV with BLT1 and Gβγ.** BV co-expressing BLT1 and various classes of Gα subunit with $G\beta_1\gamma_2$ were incubated with 0.3 nM [^{35}S]GTPγS plus 0.1 nM to 0.1 mM of GDP in the presence (■) and absence (□) of 100 nM LTB_4. Displacement of [^{35}S]GTPγS binding by GDP is shown (n = 4, mean ± S.E.). *WT*, wild type.

cles (23). We further exploited this feature by using a co-expression system with GPCR and G proteins to reconstitute the high affinity receptor. In addition, we observed that the combinatorial co-expression of individual G protein subunits could accommodate GPCR-G protein coupling specificity in terms of ligand affinity.

Sf9 cells infected with His-BLT1 baculovirus released BV on which the BLT1 receptor was expressed. The BV fraction exhibited higher ligand binding activity than the membrane fraction, whereas the membrane fraction contained an excess amount of receptor protein. These results are in good accordance with a previous report that addressed β_2-adrenergic receptor expression on extracellular virus (23). Human β_2-adrenergic receptor expressed on the BV of baculovirus exhibited specific ligand binding activity (23). β_2-Adrenergic receptor is known to couple to the G_s class of G protein (24). Because $G\alpha_s$ is abundantly present in Sf9 cells, the recovered baculovirus contained sufficient intrinsic G_s-class G protein such that evidence provided that the functional high affinity receptor was expressed in BV (23).

When BLT1 was expressed alone, the BV fraction exhibited a small number of high affinity and a large number of low affinity ligand binding sites. The K_d value of LTB_4 to BLT1 expressed on BV in the high affinity state was 0.126 nM, which is similar to that of human BLT1 expressed in Cos-7 cells previously reported (29). The high affinity and GTP-sensitive binding sites are thought to reflect receptors coupled to G proteins, whereas the low affinity sites represent uncoupled receptors. In Sf9 cells, various G protein subtypes are expressed in different amounts depending on the class of G pro-

tein (12, 46). The small number of the high affinity binding sites observed in BV expressing BLT1 alone may represent a receptor population coupled to endogenous G proteins.

In this study, we co-infected Sf9 cells with recombinant baculoviruses containing the cDNAs for BLT1 and mammalian heterotrimeric G protein ($G\alpha_{i1}\beta_1\gamma_2$). The $G\beta_1\gamma_2$ combination used here is capable of interacting with most classes of the G protein α subunit (13, 47). In each experiment, the expression of the receptor and G protein subunits was confirmed with immunoblotting (Fig. 3). The expression level of each component in recovered BV correlated well to the amount of recombinant baculovirus used to infect cells. The majority of the receptors expressed in the membrane fraction from Sf9 cells co-infected with BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ recombinant baculoviruses exhibited high affinity ligand binding (Fig. 5, *a* and *c*). In the membrane fraction, co-expression of BLT1 and $G\alpha_{i1}\beta_1\gamma_2$ increased high affinity ligand binding sites 3.6-fold compared with BLT1 alone. Even co-expression of BLT1 with $G\alpha_{i1}$ alone led to a 2.8-fold increase in the high affinity binding sites compared with that of BLT1 by itself (Fig. 5*c*, Table I). Because $G\beta$ subunits have been reported to be detected in Sf9 cells (46), the endogenous $G\beta\gamma$ subunits of Sf9 cells could well be recruited to the membrane fraction co-expressing receptor and the $G\alpha$ subunit and could reconstitute the heterotrimer with exogenous $G\alpha_{i1}$. The BV released from the co-infected cells also exhibited high affinity for ligand binding comparable with the binding affinity observed in mammalian expression systems (29) (Fig. 5, *b* and *d*). For the BV fraction, co-expression of BLT1 and $G\alpha_{i1}$ without $G\beta\gamma$ resulted in only a slight increase in the high affinity binding sites (Fig. 5, *b* and *d*), probably because BV contains a smaller amount of the endogenous $G\beta\gamma$ subunits. It was necessary for BV to express both $G\alpha_{i1}$ and $G\beta\gamma$ together with BLT1 to achieve maximum binding (Fig. 5*d*, Table I). These results are compatible with the finding that $G\beta\gamma$ complex is required for optimal receptor-G protein interaction (13, 17, 18, 47). The changes in the number of high affinity ligand binding sites on BV depend on the expression of exogenous G protein subunits. From these results, a BV co-expression system could a provide better method for the reconstitution of GPCR and G proteins because the background of the endogenous G proteins is lower than in the cell membrane fraction.

The Sf9 cell expression system has also been used to study the coupling specificity of receptors toward their cognate G proteins (14, 17). Interestingly, BLT1 BV co-expressed with $G\alpha_{oA}\beta_1\gamma_2$ as well as $G\alpha_{i1}\beta_1\gamma_2$ exhibited high affinity ligand binding (Fig. 8) and also ligand-induced GTPγS binding (Fig. 9). These data indicate that $G\alpha_{oA}\beta_1\gamma_2$ is able to couple to BLT1 and that ligand-induced activation of the receptor led $G\alpha_{oA}$ to become a GTP-bound form. Consistent with our findings, a previous report also showed that partially purified LTB_4 receptor from porcine leukocytes was able to couple with exogenous G_i-class G proteins, including $G\alpha_o$ (32). The physiological relevance of the BLT1 signaling pathway mediating $G\alpha_o$ remains to be elucidated. $G\alpha_o$ is reported to be expressed only in brain and neuronal tissues (33) and mediates neurotransmitter receptor signaling. Human BLT1 mRNA expresses mainly in peripheral leukocytes and also in spleen and thymus, albeit at low levels (29). Recently, BLT1 expression was reported in C6 astroglioma cells (48). A specific antagonist for BLT1 markedly inhibited β-amyloid-induced generation of reactive oxygen in C6 cells (48). BLT1 expression was also detected in the hippocampus of brain and other tissues in a GeneChip analysis of normal human tissues (System Biology and Medicine Data base, LSBM, RCAST, The University of Tokyo, www2.genome.rcast.u-tokyo.ac.jp data base). These observations are suggestive of a physiological function for BLT1 in the central nervous system.

The co-expression of BLT1 with any one of the other G proteins tested ($G\alpha_s$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$, $G\alpha_{12}$, and $G\alpha_{13}$ with $G\beta_1\gamma_2$ in every case) had no significant effect on ligand binding (Fig. 8). A previous report showed the $G\alpha_s$ did not couple to BLT1 in the cAMP response (29). The $G\alpha_{16}$, but not $G\alpha_{11}$, was considered to be coupled to BLT1 in inositol phosphate production (30). For the BLT1 BV co-expressing heterotrimeric G_q-class G protein, we observed a slight difference in the displacement of [^{35}S]GTPγS binding by GDP in the presence or absence of ligand (Fig. 9, *d–f*). The rank order of ligand-induced [^{35}S]GTPγS binding was $G\alpha_{16} > G\alpha_{14} \geq G\alpha_{11}$, which was comparable with the previous report (30). For the $G_{12/13}$-class G protein, it is speculated that $G\alpha_{13}$ also mediates BLT1 signaling functions such as chemotactic attraction of peripheral leukocytes. Although $G\alpha_{12}$ and $G\alpha_{13}$ belong to the same class of G proteins, they mediate different signals and generate overlapping effects (5). $G\alpha_{13}$ has been reported to directly interact with and activate p115 RhoGEF, a guanine nucleotide exchange factor for the GTPase Rho, which activates Rho signaling (49, 50). $G\alpha_{13}$-mediated Rho signaling regulates a variety of cellular responses such as chemotaxis, cell-cycle progression, and axonal guidance by controlling the organization of the actin cytoskeleton (51). In our findings presented here, BLT1 BV co-expressing $G\alpha_{13}$, rather than $G\alpha_{12}$, with $G\beta_1\gamma_2$ exhibited slightly increased [^{35}S]GTPγS binding by LTB_4 (Fig. 9, *g* and *h*). Taken together, our data demonstrate that BLT1 expressed on BV conserve the specificity of receptor-G protein coupling seen under physiological conditions. This strongly suggests this expression system should prove highly useful for the combinatorial assessment of coupling specificity of newly identified receptors to various classes of $G\alpha$ subunits.

Recently, the chemokine receptors CCR5 and CXCR4 were reported to have incorporated into murine leukemia virus particles. Murine leukemia virus particles containing chemokine receptors were attached to an optical biosensor and were utilized in antibody and human immunodeficiency virus-1 gp120 binding studies (52). Furthermore, it was possible to immobilize the baculovirus BV on a matrix slide with a density of ~1000 spots/cm^2,[2] a technological advance that led to the development of the virus chip. In a baculovirus expression system, the GPCR expressed on BV remain active for at least several months at 4 °C (data not shown), except in those rare cases of degradative inactivation. This system is considered to have several advantages over the other available expression systems in terms of application of a biochip sensor. These advantages include the availability of recombinant viruses encoding various G protein subclasses, the reconstitution of high affinity receptors by co-infection of each recombinant virus, as demonstrated here, and the low background of the intrinsic GPCR in Sf9 cells. This system is also expected to be of considerable utility in efforts to characterize the interaction of GPCR and G proteins as well as other molecules, such as the regulator of G protein signaling.

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